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Prenatal exposure to tobacco smoke sex-dependently influences methylation and mRNA levels of the *Igf* axis in lungs of mouse offspring

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Running head: *Igf1r* methylation after prenatal smoke exposure

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MNH designed the mouse experiment, analyzed the data and revised the manuscript

ABSTRACT

Background: Prenatal smoke exposure is a risk factor for abnormal lung development and increased sex-dependent susceptibility for asthma and COPD. Birth cohort studies show genome-wide DNA methylation changes in children from smoking mothers, but evidence for sex-dependent smoke-induced effects is limited.

The insulin-like growth factor (IGF) system plays an important role in lung development. We hypothesized that prenatal exposure to smoke induces lasting changes in promoter methylation patterns of *Igf1* and *Igf1r*, thus influencing transcriptional activity, and contributing to abnormal lung development.

Method: We measured and compared mRNA levels along with promoter methylation of *Igf1* and *Igf1r* and their protein concentrations in lung tissue of 30-day-old mice which had been prenatally exposed to cigarette smoke (PSE) or filtered air (control). Body weight at 30 days after birth was measured as global indicator of normal development.

Results: Female PSE mice showed lower mRNA levels of *Igf1* and its receptor (*Igf1:* $p = 0.05$; *Igf1r:* $p = 0.03$). Furthermore, CpG-site specific methylation changes were detected in *Igf1r* in a sex-dependent manner and the body weight of female offspring was reduced after prenatal exposure to smoke, while protein concentrations were unaffected.

Conclusion: Prenatal exposure to smoke induces a CpG-site specific loss of *Igf1r* promoter methylation, which can be associated with body weight. These findings highlight the sex-dependent and potentially detrimental effects of *in utero* smoke exposure on DNA methylation and *Igf1* and *Igf1r* mRNA levels. The observations support a role for *Igf1* and *Igf1r* in abnormal development.

Keywords: “epigenetics” “pyrosequencing” “asthma” “COPD” “fetal programming” “Developmental Origins of Health and Disease”

LIST OF ABBREVIATIONS

COPD	Chronic obstructive pulmonary disease
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor receptor 1
IUGR	Intrauterine growth restriction
PSE	Prenatal smoke exposure

INTRODUCTION

Maternal smoking during pregnancy has detrimental effects for offspring, such as increased risk of pulmonary diseases like as asthma (12, 13, 18) and chronic obstructive pulmonary disease (COPD) (28), but the mechanisms remain largely unknown.

Negative gestational outcomes can be caused by epigenetic alterations of which aberrant DNA methylation is commonly analyzed. It primarily refers to 5-methylcytosine which occurs when neighboring a guanidine nucleotide (CpG-site). Often applied to a gene's promoter region, this epigenetic mode can alter a its transcriptional activity.

Previous human genome-wide studies have linked prenatal smoke exposure (PSE) to alterations of DNA methylation patterns in blood samples of newborns and children (e.g., 7, 14, 15). Such alterations may play a role in abnormal fetal development and increased susceptibility for asthma and COPD. Interestingly, certain DNA methylation marks persist in prenatally exposed children (30) and alterations in DNA methylation due to smoking during pregnancy are still observed later in life. The association of maternal smoking and DNA methylation seems to be more profound in girls than in boys (6), but possible interactions of smoke exposure and the offspring's sex on methylation is rarely investigated.

The importance of the insulin-like growth factor (IGF) system to lung development, particularly *Igf1* and its receptor *Igf1r*, is highlighted in *Igf1*- or *Igf1r*-depleted mice that show a failure in lung development and diminished growth (10, 20). IGF-1, which exclusively interacts with IGF1R (21), was decreased in female but not male fetuses of asthmatic mothers who smoked during pregnancy (8) and the lower birth weight of female but not male neonates correlated with reduced IGF-1 concentrations (8). For these reasons, we chose *Igf1* and *Igf1r* as target genes for our analyses.

In lungs of 1-day-old mouse offspring, we previously found reduced mRNA levels of developmentally relevant genes after prenatal smoke exposure (3). Based on these indications,

we hypothesized that PSE negatively affects *Igf1* as well as *Igf1r*. To test this postulate, we determined the effect of prenatal smoke exposure on the protein and mRNA levels as well as promoter methylation status of *Igf1* and *Igf1r* in lungs of 30-day-old offspring. These data were related to each other and the offspring's body weight, a robust indicator of abnormal or normal development.

MATERIAL & METHODS

Animals & smoke exposure

Female Balb/c mice were obtained from Harlan (Horst, The Netherlands) at 8 to 10 weeks of age. The experimental setup was approved by the local committee on animal experimentation (DEC4575A) and carried out as described previously (4). Offspring (6 male, 5 female) of 11 smoke exposed dams together with 6 male and 8 female offspring from 15 control dams were randomly selected from each nest, weighed, and euthanized 30 days after birth for organ collection. Isolated lungs were immediately frozen in liquid nitrogen and stored at -80 °C until further use.

Quantification of IGF1 and IGF1R protein levels in lung homogenates

IGF1 and IGF1R concentrations were measured in homogenized lung tissue (the two smallest right lung lobes, as described in (4)). For quantitative determination of IGF1 concentrations, the Quantikine® ELISA Mouse/Rat IGF-I Immunoassay (R&D Systems Europe, LTD, Abingdon, UK) was used following the manufacturer’s instructions. The quantification of IGF1R was performed using the Mouse IGF1R/Igf1 receptor ELISA kit (Sandwich ELISA) (LifeSpan BioSciences, Inc., Seattle, USA) as described by the manufacturer. For IGF1 23 out of 25 samples, and for IGF1R 19 out of 25 samples had sufficient quality for analysis by ELISA.

DNA & mRNA extraction

DNA and mRNA were extracted from whole lung tissue using the AllPrep DNA/RNA Mini Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's protocol.

mRNA expression analysis

qRT-PCR for mRNA expression was performed using qPCR MasterMix Plus (Eurogentec, Seraing, Belgium) with commercially available primers for target genes *Igf1* (product number: Mm00439560_m1) and *Igf1r* (product number: Mm00802831_m1) (TaqMan® Gene Expression Assay, Applied Biosystems, Foster City, CA, USA). Detection of amplification reactions was performed using LightCycler® 480 System (Roche Diagnostics GmbH, Mannheim, Germany) with cycling conditions as follows: 50°C for 2 min, 90°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Reactions were performed in triplicate for each sample with *Hprt* (Mm03024075_m1) used for normalization. We excluded 1 out of 25 data points for *Igf1r* and 2 out of 25 for *Igf1* mRNA levels due to large differences between housekeeping and target genes.

Pyrosequencing-based bisulfite PCR analysis

In order to assess promoter methylation levels of selected genes, bisulfite sequencing primers were designed using PyroMark assay design software (version 2.0, Qiagen). Selection of CpG-sites was based on manual identification of CpG dinucleotides, using ENSEMBL genome web browser (Ensembl 83: Dec 2015) and transcript location for the identification of gene promoter regions. The mouse *Igf1* (ENSMUSG00000020053) has eight transcripts. In this study, we focused on transcript *Igf*-005 (ENSMUST00000122386, details in Discussion). The analysis of mouse *Igf1r* gene (ENSMUSG00000005533) was done by using transcript

ENSMUST00000005671.

Extracted genomic DNA from lung (200 ng) was converted with sodium bisulfite (EZ DNA Methylation-Direct™, Zymo Research, Irvine, CA) following the manufacturer's instructions. In short, the bisulfite conversion was carried out in the dark at 98 °C for 10 minutes and 64 °C for 3.5 hours followed by desulphonation of the converted DNA. Gene amplification was done using HotStarTaq® MasterMix Kit (Qiagen, Venlo, The Netherlands). Further specification on amplification conditions and primer sequences are listed in ***Table 1***. Amplification conditions: 95°C for 15 min, 94°C for 30 s, 59°C for 30 s, 72°C for 30 s, 40 cycles in a reaction volume of 25 µL. To assess DNA methylation levels of *Igf1* and *Igf1r* promoter methylation, bisulfite sequencing was performed on the PyroMarkQ24 (Qiagen) instrument. Relative levels of methylation at each CpG-site were analyzed with PyroMark Q24 2.0.6 software.

Calculations and statistical methods

Relative gene expression ($2^{-\Delta ct}$ method) as well as mean %methylation and SEM were calculated in Microsoft® Office Excel 2003. Body weight, mRNA levels, protein concentratios and DNA methylation data were tested for normal distribution of residuals and, if normally distributed, analyzed using multiple linear regression analysis (IBM® SPSS® version 22 release 22.0.0.1) in order to determine if the effect of prenatal exposure to tobacco smoke interacts with the effect of sex difference. For statistical post hoc evaluation of the subgroups, two-tailed Mann-Whitney U-test was used (GraphPad Prism 5.0 Software, SanDiegeo, CA). Correlation analysis of target gene mRNA levels, percent methylation and body weight at 30 days after birth was done using linear regression. P-values ≤ 0.05 were considered significant.

RESULTS

Sex-dependent effect of PSE on body weight at 30 days after birth

In the control group, female offspring had a significantly ($p = 0.04$) lower body weight when compared to male offspring (female: 14.6 g vs. male: 16.5 g, ***Figure 1A***). After PSE, female offspring showed a significant ($p = 0.05$) reduction in body weight compared to controls (PSE: 13.0 g vs. control: 14.6 g). This decrease was less pronounced in male offspring (PSE: 14.9 g vs. control: 16.5 g), therefore the sex-specific body weight difference was lost in the male PSE group ($p < 0.07$). An interaction of both parameters, sex and PSE, on the body weight was not seen (linear regression).

Quantification of IGF1 and IGF1R in lung homogenates

IGF1

Differences of IGF1 concentrations after PSE appeared to be more pronounced in female (control: 5965 pg/ml vs. PSE: 4885 pg/ml) than in male offspring (control: 5990 pg/ml vs. PSE: 5733 pg/ml), but did not reach statistical significance (data not shown).

Using linear regression, the variation in IGF1 concentrations contributed to the variation of the offspring's body weight by 30% ($R^2 = 0.29$, $p = 0.01$) (***Figure 1B***). This contribution was mostly derived from the prenatally smoke exposed offspring (PSE: $r = 0.86$, $p = 0.002$ vs. control: $r = 0.25$, ns). Here, the effect was more pronounced in female ($r = 0.98$, $p = 0.02$) than in male ($r = 0.80$, $p < 0.06$) offspring (***Table 2***). Over all, an association of IGF1 concentrations in whole lung tissue and the offspring's body weight, independent of the type of exposure, was found for female ($r = 0.62$, $p = 0.04$), but not for male ($r = 0.46$, ns) 30-day-old mice (***Table 2***).

IGF1R

Similar to the findings for IGF1 protein levels, also the difference in the concentration of IGF1R in lung homogenate did not reach statistical significance when comparing PSE mice to control offspring, but were higher in females (control: 5516 pg/ml vs. PSE: 5807 pg/ml) than in males (control: 4061 pg/ml vs. PSE: 4293 pg/ml; data not shown). Contrasting the observation for IGF1, the variation in IGF1R concentration did not contribute to the variation of the offspring's body weight (linear regression, $R^2 = 0.09$, ns; ***Figure 1C***)

Sex-dependent effect of PSE on mRNA concentrations of *Igf1* and *Igf1r*

Igf1

PSE reduced mRNA levels of *Igf1* in female offspring ($p = 0.05$) (***Figure 2A***), but not in male offspring. In the control groups, differences of the mRNA levels of male and female offspring were not significant ($p = 0.1$; ***Figure 2A***).

Igf1r

Figure 2B displays mRNA levels for *Igf1r*. Again, female mice showed a reduced gene expression after prenatal smoke exposure ($p = 0.03$) (***Figure 2B***), while no effect was detected in male offspring. Notably, higher base line mRNA levels were seen in female offspring but did not reach statistical significance ($p = 0.07$; ***Figure 2B***).

Using linear regression, no interaction of parameters, sex and PSE, was seen for both mRNA levels. However, it revealed a strong positive correlation of mRNA levels between both genes ($R^2 = 0.91$, $p < 0.001$; ***Figure 3A***).

Igf1 gene expression and protein concentrations were only seen to correlate in female PSE, but not in male offspring (linear regression, female: $R^2 = 0.90$, $p = 0.05$ vs. male: $R^2 < 0.01$, ns) wherefore a correlation in all offspring was not seen (***Figure 3B***).

Igf1r gene expression, on the other hand, correlated with IGF1R protein concentrations (linear regression; $R^2 = 0.35$, p = 0.02; ***Figure 3C***). This effect appears to originate from female offspring (r = 0.72, p = 0.05; ***Table 3***), predominantly from female control animals (r = 0.93, p = 0.02; ***Table 3***) while in male offspring no association was seen (r = 0.10; ns).

Similarly, the variation in gene expression of *Igf1* and *Igf1r* in control animals contributed to their variation in body weight by 62% and 69%, respectively (linear regression, *Igf1:* p = 0.002; *Igf1r*: p = 0.002; ***Tables 2&3***), which was also seen for female but not for male offspring (*Igf1*: female: r = -0.76, p = 0.05 vs. male: r = -0.52, ns, ***Table 2***; *Igf1r*: female: r = -0.86, p = 0.03 vs. male: r = -0.69, ns; ***Table 3***).

Effect of PSE on promoter methylation of *Igf1* and *Igf1r*

Igf1

Figure 4 illustrates the mean percent methylation of each analyzed CpG-site in the promoter of *Igf1*. The targeted promoter region of *Igf1* did not reveal differences in methylation levels in any of the analyzed CpG-sites after prenatal smoke exposure. ***Figure 5*** provides a sex-specific overview of CpG-site specific data points of *Igf1*, which does not show additional significant findings.

A linear relationship was found between protein concentrations and methylation status of CpG-1509 in all control animals (r = -0.79, p = 0.001). Here, the effect was more pronounced in female than in male offspring (female: r = -0. 93, p = 0.002 vs. male: r = -0.79, p = 0.06; ***Table 2***). This observation is contrasted by a linear relation for all control animals at CpG-1212 (r = 0.64, p = 0.02), which was found in male but not in female offspring (male: r = 0.83, p = 0.04 vs. female: r = 0.13, ns; ***Table 2***). For that same CpG-site also a linear relation was found for *Igf1* mRNA concentrations in all control animals (r = -0.60, p = 0.04). This observation was again sex-dependent, as it was only seen in female but not in male

control mice (female: r = -0.85, p = 0.02 vs. male: r = -0.46, ns; ***Table 2***).

Moreover, only for the female PSE group a trend for a linear relationship was found between the methylation status at CpG-1180 and protein concentrations (r = 0.93, p = 0.07; ***Table 2***) as well as body weight (r = 0.86, p = 0.06; ***Table 2***).

Igf1r

The mean percent methylation of *Igf1r*'s promoter region is depicted in ***Figures 6,*** while a sex-specific overview of CpG-site specific data points is provided in ***Figures 7 and 8***. The analysis of *Igf1r* promoter allowed three observations:

Firstly, a sex-independent reduction was found for the %methylation of *Igf1r* CpG-272 (p = 0.04, ***Figure 6***) together with a trend for lower methylation status after prenatal smoke exposure at CpG-252 (p = 0.08). Within the entire PSE group, significant correlations (linear regression) were seen for mRNA concentrations with % methylation at CpG-201 (r = 0.67); protein concentrations with CpG-249 (r = 0.78) and CpG-194 (r = 0.92) as well as body weight with CpG-233 (r = 0.62) and CpG-206 (r = -0.66; ***Table 3***).

Secondly, a sex-dependent reduction in methylation levels was found at *Igf1r* CpG-233 for male (p = 0.04) and female (p = 0.05) offspring when compared to their control groups (***Figure 7A***). The methylation status of female PSE offspring at this CpG-site was significantly lower when compared with male PSE offspring (p = 0.04). Notably, at *Igf1r* CpG-206 on the other hand, prenatally smoke exposed female offspring showed higher CpG-site specific methylation when compared to male PSE mice (p = 0.02) (***Figure 8***).

Thirdly, within all analyzed offspring, linear regression revealed a correlation of promoter methylation and mRNA concentrations at CpG-201 (r = 0.62) and -17 (r = 0.55). This observation was augmented in the male group (r = 0.76 and r = 0.65, respectively; ***Table 3***). Moreover, in all analyzed offspring, protein concentrations were seen to correlate

with methylation status at CpG-201 (r = 0.45), CpG-194 (r = 0.49) and CpG-171 (r = 0.51; ***Table 3***) of which the correlation seen for CpG-194 was enhanced in PSE offspring (r = 0.92). Interestingly, linear regression also uncovered that the methylation status at *Igf1r* CpG-233 contributed to the variation of body weight at 30 days after birth by 30% ($R^2 = 0.30$, p = 0.004; ***Figure 7B***). This effect was also seen, sex-independently, for the PSE mice (r = 0.62, p = 0.04; ***Table 3***).

DISCUSSION

According to the "fetal origins of disease" hypothesis (1, 2), an adverse fetal environment has long lasting consequences for the offspring. In this study we investigated the effect of prenatal smoke exposure (PSE) on mRNA and DNA methylation levels of *Igf1* and *Igf1r* as well as their protein concentrations in lungs of 30-day-old mouse offspring. Our results support the hypothesis that smoking during pregnancy affects mRNA levels of *Igf1* and *Igf1r* in a sex-dependent way.

Smoking during pregnancy has a negative effect on the birth weight of a newborn. In our mouse model, we use the body weight at 30 days after birth as a global indicator of abnormal prenatal development. Apart from the *in utero* smoke exposure, housing conditions of all animals were identical. Alterations of the body weight are therefore likely to be caused by the experimental variable, here prenatal smoke exposure. Both male and female offspring showed a reduction of approximately 10% in body weight at 30 days after birth from smoke-exposed mothers compared to their matching control group. Similar findings were described in mice following prenatal smoke exposure by Larcombe et *al.* (16).

In humans, prenatal smoke exposure has previously been linked to intrauterine growth restriction (IUGR) which in turn was shown to increase the risk of developing asthma (35). Moreover, disrupted prenatal lung development was linked to the development of COPD later

in life (9). Protein concentrations of IGF1 were reduced in cord plasma of babies born to mothers who had smoked during pregnancy, which may have contributed to fetal IUGR (29). This suggests that prenatal smoke exposure might have some attenuating effects on growth due to reduced IGF1 or a deranged IGF signaling pathway. While no significant effect of prenatal smoke exposure on IGF1 and IGF1R protein levels was detected, we found a strong correlation of IGF1 and the offspring's body weight, in particular in the PSE females. Furthermore, protein levels for IGF1R correlated with *Igf1r* gene expression, which again was found for female offspring exclusively. These data are accompanied by the reduced mRNA levels for *Igf1* and *Igf1r* seen in lungs of 30-day-old offspring after prenatal smoke exposure.

The simultaneous reduction of mRNA levels for both *Igf1* and *Igf1r* after PSE and hence their strong positive correlation could be explained by a negative feedback loop. This was also proposed by Moreno-Barriuso et *al.*, who suggested that IGF1 could regulate the expression of its own receptor (22).

Given the abrogated lung maturation after *Igf1* and/or *Igf1r* gene deletion (10, 20), the observed reduction of *Igf1* and *Igf1r* gene expression in lungs of 30-day-old mice may reflect abnormal lung development, as at this age the alveolar phase is ending. Although in these mice neither the actual asthma phenotype nor the allergen susceptibility of the 30-day-old offspring was assessed, it is conceivable that a repression of important signaling cascades, such as IGF, during developmental stages of the lung could have long(er) lasting effects later in life.

The *Igf1* gene contains 6 exons (23) and so far at least nine different *Igf1* isoforms are known (33). These are generated by utilizing different promoters and splicing variation. Transcripts comprising the first exon are referred to as Class1, those with the second exon as Class2 transcripts (23). Class2 depleted mice did not show an affected viability or phenotypical changes, but a compensatory up-regulation of Class1 transcripts (33). Consequently, we

investigated a possible effect of PSE on P1 promoter methylation of the Class1 *Igf1* isoform. Our assessment did not reveal any smoke induced effects in 30-day-old mouse offspring but associations of body weight, mRNA levels and protein concentrations with methylation status were found by linear regression. Interestingly, these associations were seen when distinguishing by the offspring's sex and their prenatal exposure. However, the active expression of genes requires an orchestration of many (co-) factors of which DNA methylation can be one out of several epigenetic modes. The lack of a direct correlation was anticipated as the mechanistic link between DNA (de)methylation and gene silencing or activation is complex. Other epigenetic modes (i.e., histone modifications, chromatin remodeling and RNA-based mechanisms (lncRNAs / miRs)) seem to be interlinked, but their chronological order and the exact mechanism(s) that may connect these modes still need to be described to their full extent (reviewed by e.g., 31).

Female offspring showed a stronger PSE-induced reduction of *Igf1* as well as *Igf1r* transcripts when compared to male offspring. The implication of a role for sex hormones influencing the expression of *Igf1* isoforms is in discussion for a long time and indeed, Class1 transcripts responded to a higher degree to estrogen activation than Class2 transcripts (25). Cord plasma/blood concentrations of IGF1 in female neonates were seen higher than in males (11, 36) and a dimorphic expression pattern was suggested. Similarly, we found a trend for higher baseline levels of *Igf1* and *Igf1r* mRNA levels in females when comparing them to male control offspring. Recently, in a COPD mouse model, female mice showed, compared to male mice, an increased morphologic remodeling of the small airways after six months of cigarette smoke exposure (32). These observations suggest either a higher vulnerability of female mice to prenatal insults such as cigarette smoke exposure, or the availability of quicker and/or more efficient compensatory mechanisms to counteract any insult either on a prenatal or early

postnatal stage in male mice. This is of interest, as also in humans, the prevalence rate for COPD is higher in women than in men (3.5% vs. 2.9%) (24).

In contrast to the findings for *Igf1*, *Igf1r* promoter methylation was altered after PSE at three CpG-sites. Even though the base line methylation levels at CpG-206 of male and female control groups were similar, after PSE hypomethylation was seen in male and hypermethylation in female offspring, which suggests a possible sex-dependent response to PSE. Similarly, *Igf1r* mRNA concentrations correlated with methylation status at two CpG-sites (CpG-201 and CpG-17) and in both cases, this association originated from male offspring with an additional contribution of the PSE mice. Moreover, *Igf1r* CpG-233 was detected to be hypermethylated after PSE and could be linked to the offspring's body weight at 30 days after birth. Interestingly, this can only be seen independently of the offspring's sex, but was also found in the group of prenatally smoke exposed offspring. Lastly, also *Igf1r* CpG-272 showed sex-dependent hypomethylation. Here, PSE caused a loss of correlation to *Igf1r* mRNA levels but induced a correlation to the offspring's body weight, predominantly in male mice.

Epigenetic marks, such as DNA methylation, are shown to be affected by prenatal smoke exposure and can increase the risk of developing asthma in mice and men (16, 37). The PSE-responsive CpG-sites of the *Igf1r* promoter region suggest a role for DNA methylation in the expression of *Igf1r* and its relevance in mediating the IGF system; determining to which extent however, requires further studies.

The majority of DNA methylation studies links promoter hypermethylation with gene silencing and the lack of methylation with gene "activation". However, several recent studies report upon a positive association of DNA methylation status and gene expression (i.e., 5, 19). Other studies find that the link between DNA methylation and gene expression depends on

where in the gene sequence the methylation occurs/is detected (gene body vs. flanking regions, transcription start site, 5'-untranslated region etc.). Taking together, these findings suggest that the relation of DNA methylation and gene expression may not be as strict as previously described.

In light of these findings and the complex network of several epigenetic modes, we conclude from our observations that differential CpG-site specific methylation after PSE may depend on the offspring's sex.

We recognize that we analyzed only one promoter region for the *Igf1* gene and it could very well be that by doing so, we missed other putative methylation sites. One limitation of our data is the lack of methylation information on other potentially relevant parts of the *Igf1r* sequence (7 CpG-sites, CpG-146 to -104). Future analysis of this and other potentially regulatory regions is warranted. Nevertheless, methylation changes at CpG-site resolution, as we found for *Igfr*-233, can be functionally important.

Other studies indicated that "CpG-137" and "CpG-611" of the human *Igf1* gene may have functional relevance, as they were found to contribute to height and serum IGF1 variation in PBMCs of pre-puberty children (26). Additionally, the impact of *Igf1* "CpG-137" methylation on serum IGF1 level variation seems to increase in children with idiopathic short stature after treatment with growth hormone (27). Moreover, one CpG-site of the *Igf1r* gene (cg12562232) was significantly associated with differences in birth weight of monozygotic twins (34).

To our knowledge, this is the first study to demonstrate effects of *in utero* smoke exposure on *Igf1r* and *Igf1* promoter methylation and mRNA levels in mouse lungs. These findings emphasize the sex-dependent effects of PSE and indicate a role of the IGF system, represented here by *Igf1* and *Igf1r*, in (lung) development in mouse offspring. Even though

studies could link decreased serum IGF1 levels in the fetal circulation with maternal smoking during pregnancy (e.g., 29), a sex-dependent distinction is rarely done. Notably, maternal smoking was associated with reduced *IGF2* methylation in DNA of umbilical cord white blood cells, with a stronger effect in newborn girls than boys (6). Also, Richmond et *al*. found sex-specific associations for DNA methylation changes in the offspring's cord blood, when compared with non-smokers. Of these associations, one CpG-site at AHRR (cg05575921) was found with a smoke-induced effect larger in girls than in boys whereas another CpG-site at CYP1A1 (cg05549655), the observed effect was larger in boys than in girls (30).

In summary, evidence for a sex-specific effect of maternal smoking during pregnancy can be found in human studies, but it is limited. Our data indicate that sex-differences in maternal smoking effects need more attention and may provide important insights into pathogenesis of health effects. Furthermore, the present study provides a sex-specific link between prenatal smoke exposure, epigenetic modifications, body weight, gene expression and protein levels. This information may be used to identify future targets for therapeutic intervention.

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529 **DISCLOSURES**

530

FIGURE CAPTIONS

Figure 1. Body weight [g] comparing control (○) with prenatally smoke exposed (●) offspring and correlations of body weight with IGF1 and IGF1R concentrations in whole lung tissue.

A) Prenatally smoke exposed (PSE) and air exposed control mice were euthanized at 30 days after birth. Prior to euthanasia, the body weight [g] was assessed (individual values per group and sex, median depicted as a horizontal line).

B) The body weight [g] of all offspring correlates with IGF1 concentrations in whole lung (linear regression).

C) A correlation of body weight [g] with IGF1R concentrations in whole lung was not found.

If not stated otherwise, the comparison of displayed groups was not significant.

Figure 2. *Igf1* and *Igf1r* mRNA levels comparing 30d control (○) with prenatally smoke exposed (●) offspring.

A) *Igf1* and B) *Igf1r* mRNA levels were measured in whole lung tissue of 30-day-old prenatally smoke exposed and control mice and corrected for housekeeping gene *Hprt*. A sex-specific reduction of mRNA levels was seen in female mice for both genes, whereas male offspring did not show an effect of prenatal smoke exposure on mRNA levels. Data are presented per sex and group as individual values with median as a horizontal line. If not stated otherwise, the comparison of displayed groups was not significant.

Figure 3. Correlation of *Igf1* with *Igf1r* mRNA levels in lungs of 30-day-old offspring and correlations of *Igf1* and *Igf1r* gene expression with protein levels.

A) Prenatally smoke exposed (PSE) and air exposed control mice were euthanized at 30 days

after birth. Displayed mRNA levels of *Igf1* and *Igf1R* were measured in whole lung tissue and displayed as uncorrected data points. Linear regression revealed a positive relation of mRNA levels between both genes and strong interactions of both mRNA levels were found sex-dependently for all possible groups.

B) Based on linear regression, no correlation was found for *Igf1* gene expression and protein levels in lung homogenates.

C) *Igf1r* gene expression correlated with the amount of protein in lung homogenates. This effect was also seen for all female offspring and was most pronounced in the female control group.

Figure 4. Methylation of each analyzed CpG-site in the *Igf1* promoter (mean ± SEM) comparing 30d control (○) with prenatally smoke exposed (●) offspring.

DNA from lungs of 30-day-old offspring who were either prenatally smoke exposed (n = 11) or in the control group (n = 14) was assessed for *Igf1* promoter methylation status. No differences were detected. Data are shown as mean ± SEM. CpG-site annotations are relative to ATG start codon. If not stated otherwise, the comparison of displayed groups was not significant.

Figure 5. Methylation of each analyzed CpG-site in the *Igf1* promoter comparing 30d control (○) with prenatally smoke exposed (●) offspring.

DNA of lungs from 30-day-old offspring of PSE and control groups was subjected to bisulfite sequencing-based methylation analysis of *Igf1* promoter region. Data of the 8 targeted CpG-sites are presented per sex and group as individual values with median as a horizontal line. CpG-site annotations are relative to ATG start codon. If not stated otherwise, the comparison of displayed groups was not significant.

Figure 6. Methylation of each analyzed CpG-site in the *Igf1r* promoter (mean ± SEM) comparing 30d control (○) with prenatally smoke exposed (●) offspring.

Igf1r promoter methylation levels were assessed in lungs of prenatally smoke exposed (PSE) offspring (n = 11) and compared to control offspring (n = 14). Data are shown as mean ± SEM. * $p < 0.05$. CpG-site annotations are relative to ATG start codon. If not stated otherwise, the comparison of displayed groups was not significant.

Figure 7. Sex-specific methylation status and correlation of *Igf1r* CpG-233 comparing 30d control (○) with prenatally smoke exposed (●) offspring.

A) Prenatal smoke exposure (PSE) induced reduction of *Igf1r* CpG-233 in male and female offspring. Methylation status of *Igf1r* CpG-233 in female PSE offspring is significantly lower than in male PSE offspring.

B) The methylation status of *Igf1r* CpG-233 correlated positively with the offspring's body weight [g] at 30 days after birth. Data are shown as individual values. CpG-site annotations are relative to ATG start codon. If not stated otherwise, the comparison of displayed groups was not significant.

Figure 8. Methylation of each analyzed CpG-site in the *Igf1r* promoter comparing 30d control (○) with prenatally smoke exposed (●) offspring.

DNA of lungs from 30-day-old offspring of PSE and control groups was subjected to bisulfite sequencing-based methylation analysis of *Igf1r* promoter region. Data of the targeted CpG-sites are presented per sex and group as individual values with median as a horizontal line. CpG-site annotations are relative to ATG start codon. If not stated otherwise, the comparison of displayed groups was not significant.

608 **Table 1. Bisulfite amplification (F/R) and sequencing (S) primers**

Gene	Targeted CpG-site position	Sequences 5' – 3'
Igf1	1509-1430 **Amplicon length [bp]** 209	F: AGAGGGTTGGAAAGAGTTTAAG R: AAACCAAACTTACCTCAATCTCTTAC S1: AGGTTTTTATTTATGGGG S2: GTATTTTTAAATTTTTTTGAGA Sequence to analyze: S1: TAGYGTAAAGAGGTAGTGTAGAGTTTTTAATTGGTTTTTGTTTTTATYGATGTGTTAGTATTTTTAAATTTTTTTGAGA S2: GTTYGAGAGAGTAAGAGATTGAGGTAAGT
	1357-1254 **Amplicon length [bp]** 212	F: AGAGTAAGAGATTGAGGTAAGTT R: TTACCACAAAAAATAAAATTCTAATCTTC S1: GGGAAAGTATATTTGGAG S2: TTATTGAGAAATAGGTATAAAT Sequence to analyze: S1: AGATATTYGTGGAAAGTATGTAGYGTTTAATTTGGGTTTTTGTAATTTTTTTTTATAATTTATTTTTTTATTTATTGTTTTTGAAAGATTATTGAGAAATAGGTATAAAT S2: YGTATTAATAGAAGATTAGAATTTTA
	1212-1180 **Amplicon length [bp]** 250	F: TTGGAGAGATATTAGTGGAAAGTATGTAG R: AATTATAATATCATTCAAATCCCTCAACT S: AGAATTTTATTTTTTGTGGTAAAG Sequence to analyze: GYGAGTTTATATATTATAAATAGTAGAAGTAGTYGGTTTGAATTATGTTGTTAGTTATT
Gene	**Targeted CpG-site position**	**Sequences 5' – 3'**
Igf1r	272-164 **Amplicon length [bp]** 327	F: GGGGATTTTTTTTAGGAGTTAGATTTTA R: ATTTTCCTCCTTCTTCTACATCT S1: TTA TTT GGG ACG AAA TTT S2: GATAAGGAGGGTGG S3: GGAGTYGGGAAGT Sequence to analyze: S1: TTTTTATTTTYGTTTAAAAATAAGAGYGTAGGYGAYGATTTTYGGAAAGYGGYGTGGATAAGGAGGGGTGG S2: YGYGGGGYGGTTTTTTAGYGTYGGTAGTAGYGGTTTAYGGGGYGGYGGAGTYGGGAAGT S3: YGGGGYGYGTYGGGGYGGGTTGTYGGYGTYGTYGGTTTTTATTTGTAAAYGTAGAGATGTAGAAGAAGGAGGAAA
	17 **Amplicon length [bp]** 120	F: AGTGAGGATTGAGTTGGAGATTT R: CCTCCCAAACCAAACTTCATTCCTTTTAT S: ATTTTTGAGAAAAGGGAATT Sequence to analyze: TYGTTTTAAATAAAAGGAATGAAGTTT

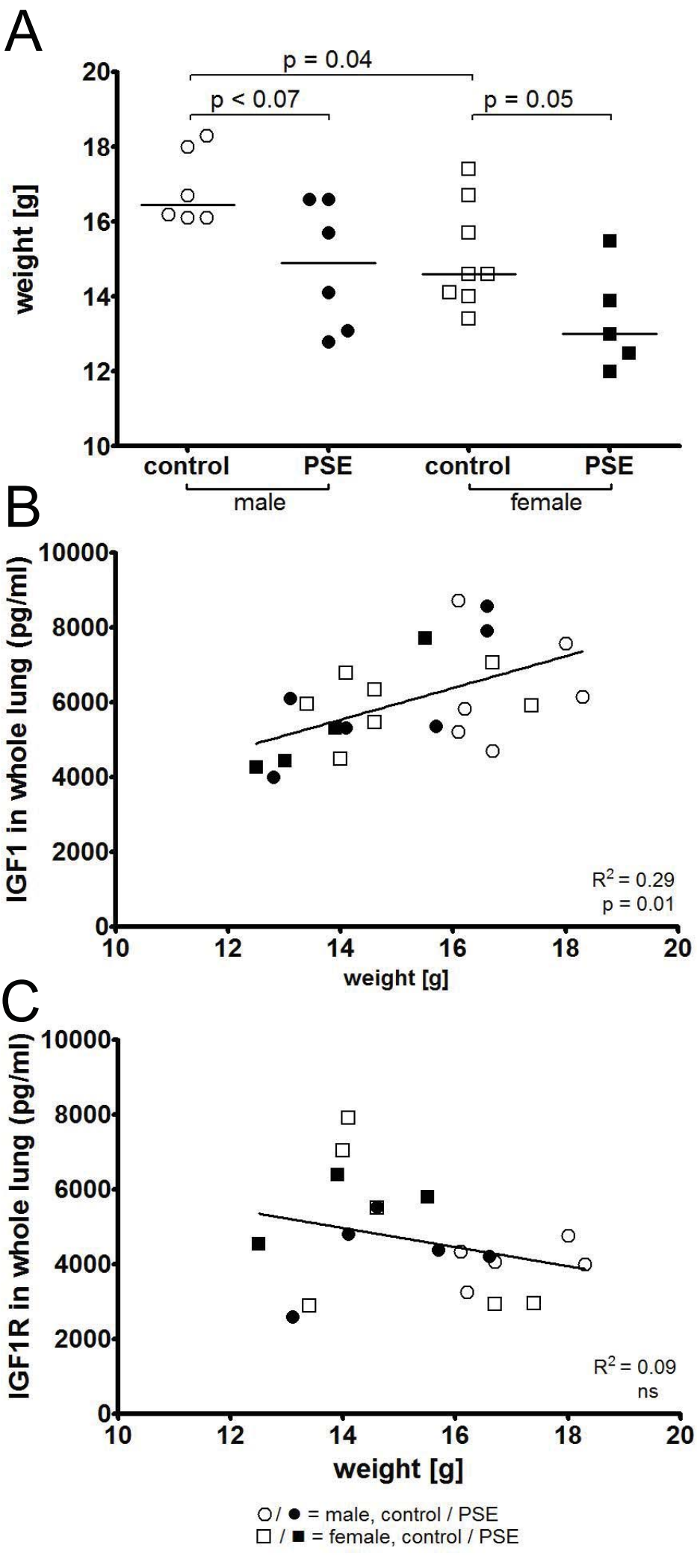
Table 2. Correlations between IGF1 protein concentrations, *Igf1* mRNA levels, *Igf1* promoter methylation and the offspring's body weight

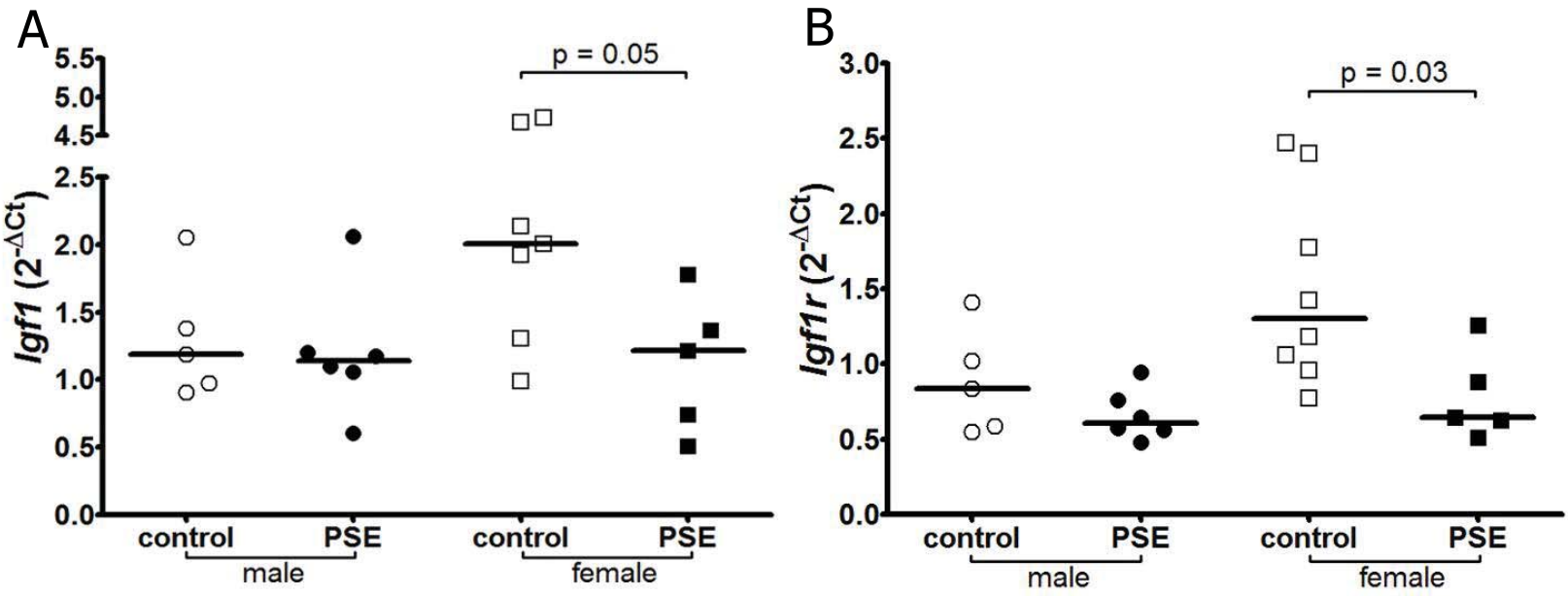
correlation of / with	IGF1 protein [pg/ml]		all all	all male	all female	all control	all PSE	male control	male PSE	female control	female PSE
***Igf1* (2-dCT)**		r	0.02	-0.02	0.18	-0.22	0.44	0.16	0.08	-0.26	**0.95**
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	**0.05**
weight [g]		r	**0.54**	0.46	**0.62**	0.25	**0.86**	0.10	0.80	0.33	**0.98**
		p-value	**0.01**	ns	**0.04**	ns	**0.002**	ns	0.06	ns	**0.02**
***Igf1* promoter methylation [%]**	CpG-1509	r	-0.29	-0.28	-0.41	**-0.79**	0.11	-0.79	0.15	**-0.93**	0.03
		p-value	ns	ns	ns	**0.001**	ns	0.06	ns	**0.002**	ns
	CpG-1465	r	-0.06	-0.19	0.26	-0.27	0.15	-0.50	0.20	0.24	0.28
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-1430	r	-0.17	-0.33	-0.02	-0.27	-0.09	-0.10	-0.44	-0.60	0.52
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-1357	r	-0.09	-0.13	-0.12	-0.54	0.29	-0.79	0.38	-0.38	-0.25
		p-value	ns	ns	ns	0.06	ns	0.06	ns	ns	ns
	CpG-1341	r	0.28	0.40	0.06	0.51	0.17	0.46	0.50	0.65	-0.37
		p-value	ns	ns	ns	0.07	ns	ns	ns	ns	ns
	CpG-1254	r	-0.29	-0.18	-0.45	-0.42	-0.13	-0.17	-0.19	**-0.83**	0.05
		p-value	ns	ns	ns	ns	ns	ns	ns	**0.02**	ns
	CpG-1212	r	0.26	0.49	-0.11	**0.64**	-0.04	**0.83**	0.23	0.13	-0.03
		p-value	ns	ns	ns	**0.02**	ns	**0.04**	ns	ns	ns
	CpG-1180	r	0.33	0.24	0.58	0.28	0.39	0.42	0.10	0.13	0.93
		p-value	ns	ns	0.08	ns	ns	ns	ns	ns	0.07
correlation of / with	***Igf1* (2-dCT)**		all all	all male	all female	all control	all PSE	male control	male PSE	female control	female PSE
weight [g]		r	-0.17	-0.32	0.01	**-0.79**	0.00	-0.52	-0.50	**-0.76**	-0.60
		p-value	ns	ns	ns	**0.002**	ns	ns	ns	**0.05**	ns
***Igf1* promoter methylation [%]**	CpG-1509	r	0.08	0.22	0.07	-0.14	0.29	-0.10	0.51	0.51	-0.21
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-1465	r	-0.27	-0.33	-0.41	-0.54	0.04	-0.61	-0.24	-0.71	0.43
		p-value	ns	ns	ns	0.07	ns	ns	ns	0.07	ns
	CpG-1430	r	0.02	-0.15	0.22	0.14	0.20	-0.81	0.12	0.50	0.32
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-1357	r	0.13	0.19	0.16	0.15	0.16	0.49	0.01	0.13	0.25
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-1341	r	-0.03	**0.60**	-0.21	-0.01	0.29	0.75	0.74	-0.43	-0.05
		p-value	ns	**0.05**	ns	ns	ns	ns	0.09	ns	ns
	CpG-1254	r	0.00	-0.28	0.03	0.20	-0.32	0.03	-0.52	0.15	-0.10
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-1212	r	-0.39	-0.42	-0.53	**-0.60**	-0.40	-0.46	-0.60	**-0.85**	-0.25
		p-value	0.07	ns	0.07	**0.04**	ns	ns	ns	**0.02**	ns
	CpG-1180	r	0.02	-0.26	0.02	0.03	0.18	-0.30	-0.20	-0.31	0.71
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
correlation of / with	**weight [g]**		all all	all male	all female	all control	all PSE	male control	male PSE	female control	female PSE
***Igf1* promoter methylation [%]**	CpG-1509	r	-0.16	-0.13	-0.39	0.05	-0.25	0.37	-0.34	-0.44	-0.07
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-1465	r	0.09	0.23	0.00	0.02	-0.17	-0.19	0.15	0.04	-0.23
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-1430	r	-0.17	-0.29	-0.32	-0.06	-0.10	0.39	-0.41	-0.59	0.27
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-1357	r	-0.02	0.05	-0.14	0.06	-0.05	-0.23	0.41	0.11	-0.62
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-1341	r	-0.05	-0.28	0.07	0.32	-0.14	-0.07	0.04	**0.77**	-0.64
		p-value	ns	ns	ns	ns	ns	ns	ns	**0.03**	ns
	CpG-1254	r	-0.04	0.11	-0.13	-0.01	0.12	**0.85**	-0.03	-0.34	0.43
		p-value	ns	ns	ns	ns	ns	**0.03**	ns	ns	ns
	CpG-1212	r	0.26	0.51	0.03	0.44	0.06	0.18	0.43	0.45	0.18
		p-value	ns	0.09	ns	ns	ns	ns	ns	ns	ns
	CpG-1180	r	0.19	0.16	0.38	0.15	0.32	**0.89**	0.03	-0.07	0.86
		p-value	ns	ns	ns	ns	ns	**0.02**	ns	ns	0.06

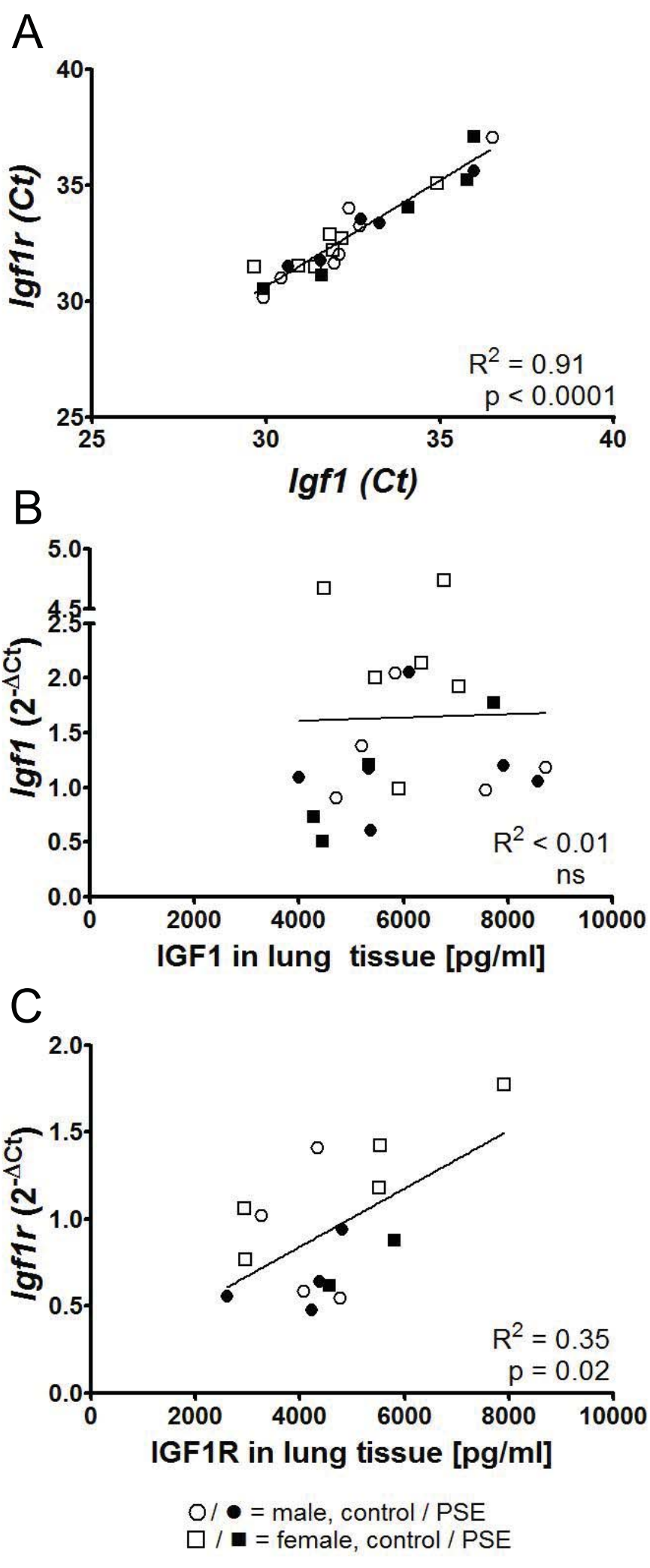
Table 3. Correlations between IGF1R protein concentrations, *Igf1r* mRNA levels, *Igf1r* promoter methylation and the offspring's body weight

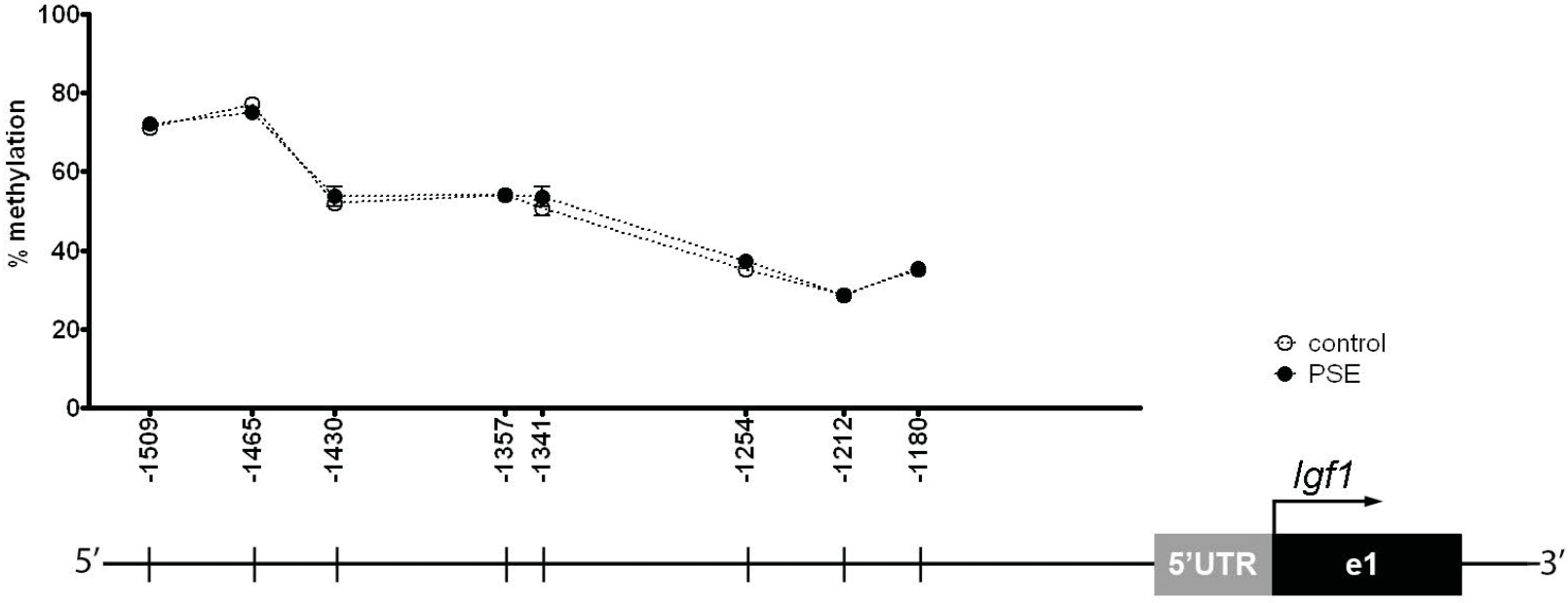
correlation of / with	IGF1R protein[pg/ml]		all all	all male	all female	all control	all PSE	male control	male PSE	female control	female PSE
***Igf1r* (2-dCT)**		r	**0.59**	0.10	**0.72**	0.65	0.66	-0.26	0.55	**0.93**	1.00
		p-value	**0.02**	ns	**0.05**	0.06	ns	ns	ns	**0.02**	Perfect line
weight [g]		r	-0.30	0.42	-0.40	-0.49	0.17	0.44	0.56	-0.54	0.64
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
***Igf1r* promoter methylation [%]**	CpG-272	r	-0.01	0.26	-0.15	0.02	-0.15	0.59	-0.36	-0.08	-0.09
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-255	r	0.25	0.20	0.29	0.31	0.28	0.24	0.79	0.40	-0.87
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-252	r	0.23	0.27	0.21	0.24	0.71	0.49	0.40	0.29	0.31
		p-value	ns	ns	ns	ns	0.07	ns	ns	ns	ns
	CpG-249	r	0.13	0.37	-0.03	0.06	**0.78**	0.67	0.27	-0.03	0.85
		p-value	ns	ns	ns	ns	**0.04**	ns	ns	ns	ns
	CpG-246	r	0.18	0.21	0.19	0.21	0.21	0.49	-0.29	0.26	-0.21
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-238	r	-0.02	0.36	-0.32	-0.15	0.55	0.72	0.16	-0.30	-0.99
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-233	r	-0.14	0.47	-0.32	-0.19	0.11	0.48	0.82	-0.26	-0.58
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-230	r	-0.25	0.05	-0.43	-0.26	-0.20	0.78	-0.81	-0.41	-0.71
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-228	r	-0.05	0.37	-0.18	-0.08	0.10	0.86	-0.05	-0.19	0.27
		p-value	ns	ns	ns	ns	ns	0.06	ns	ns	ns
	CpG-223	r	-0.02	0.06	-0.02	-0.06	0.17	0.68	-0.93	-0.12	0.83
		p-value	ns	ns	ns	ns	ns	ns	0.07	ns	ns
	CpG-215	r	0.14	0.61	0.02	0.09	0.51	0.39	0.71	0.04	0.64
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-209	r	0.36	0.55	0.35	0.31	0.63	0.39	0.72	0.34	0.77
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-206	r	0.16	0.02	-0.01	0.02	0.61	0.45	-0.78	-0.11	0.74
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-201	r	**0.45**	-0.02	0.48	0.44	0.66	-0.14	0.11	0.50	0.88
		p-value	**0.05**	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-194	r	**0.49**	0.29	0.49	0.35	**0.92**	0.20	0.86	0.42	0.99
		p-value	**0.04**	ns	ns	ns	**0.003**	ns	ns	ns	ns
	CpG-185	r	0.21	0.41	0.10	0.16	0.65	0.23	0.70	0.11	0.68
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-182	r	0.07	0.27	-0.09	-0.11	0.35	0.42	0.31	-0.57	0.67
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-171	r	**0.51**	-0.17	0.58	0.55	0.50	0.19	-0.64	0.62	0.73
		p-value	**0.03**	ns	ns	0.08	ns	ns	ns	ns	ns
	CpG-166	r	0.23	0.30	0.19	0.15	0.41	0.56	0.13	0.08	0.67
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-164	r	0.36	0.05	0.33	0.25	0.61	0.30	-0.17	0.22	0.88
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-17	r	0.21	-0.29	0.43	0.28	-0.04	-0.66	0.10	0.57	0.30
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
correlation of / with	***Igf1r* (2-dCT)**		all all	all male	all female	all control	all PSE	male control	male PSE	female control	female PSE
weight [g]		r	-0.02	-0.10	0.23	**-0.83**	-0.07	-0.69	-0.51	**-0.86**	0.80
		p-value	ns	ns	ns	**0.002**	ns	ns	ns	**0.03**	ns
***Igf1r* promoter methylation [%]**	CpG-272	r	-0.08	-0.13	0.02	**-0.64**	0.09	-0.76	0.53	-0.36	-0.12
		p-value	ns	ns	ns	**0.03**	ns	ns	ns	ns	ns
	CpG-255	r	-0.13	-0.11	-0.03	-0.54	0.22	-0.62	0.56	-0.15	-0.27
		p-value	ns	ns	ns	0.09	ns	ns	ns	ns	ns
	CpG-252	r	-0.17	-0.21	-0.08	**-0.66**	0.28	-0.80	0.26	-0.34	0.58
		p-value	ns	ns	ns	**0.03**	ns	ns	ns	ns	ns
	CpG-249	r	-0.13	-0.01	-0.13	**-0.68**	0.43	-0.72	0.41	-0.54	0.83
		p-value	ns	ns	ns	**0.02**	ns	ns	ns	ns	ns
	CpG-246	r	-0.18	-0.17	-0.12	**-0.71**	0.12	-0.80	0.54	-0.47	-0.22
		p-value	ns	ns	ns	**0.01**	ns	ns	ns	ns	ns
	CpG-238	r	-0.05	0.14	-0.16	-0.49	0.40	-0.22	0.35	-0.64	0.49
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-233	r	-0.13	0.04	-0.02	**-0.64**	0.22	-0.37	0.11	-0.77	0.65
		p-value	ns	ns	ns	**0.03**	ns	ns	ns	0.07	ns
	CpG-230	r	-0.21	-0.07	-0.17	-0.57	0.10	-0.42	0.00	-0.52	0.26
		p-value	ns	ns	ns	0.07	ns	ns	ns	ns	ns
	CpG-228	r	-0.12	-0.07	-0.12	-0.50	0.22	-0.67	0.53	-0.47	-0.29
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-223	r	-0.08	0.03	-0.02	-0.37	0.12	-0.09	-0.28	-0.49	0.80
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-215	r	0.33	0.37	0.27	0.14	0.21	0.64	0.29	-0.05	0.09
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-209	r	0.10	0.45	-0.07	0.07	0.26	0.16	**0.81**	0.15	-0.26
		p-value	ns	ns	ns	ns	ns	ns	**0.05**	ns	ns
	CpG-206	r	0.09	0.25	-0.17	-0.03	0.00	-0.06	0.29	-0.19	-0.31
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-201	r	**0.62**	**0.76**	0.52	0.57	**0.67**	0.72	0.77	0.49	0.45
		p-value	**0.003**	**0.01**	ns	0.07	**0.03**	ns	0.07	ns	ns
	CpG-194	r	-0.11	-0.22	-0.23	-0.08	0.18	-0.63	0.31	0.16	0.00
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-185	r	0.22	0.53	0.04	0.24	0.09	0.60	0.44	0.07	-0.19
		p-value	ns	0.09	ns	ns	ns	ns	ns	ns	ns
	CpG-182	r	-0.06	0.08	-0.21	-0.08	-0.20	0.24	0.17	**-0.89**	-0.73
		p-value	ns	ns	ns	ns	ns	ns	ns	**0.05**	ns
	CpG-171	r	0.10	0.57	-0.16	0.10	-0.04	0.68	0.32	0.09	-0.74
		p-value	ns	0.07	ns	ns	ns	ns	ns	ns	ns

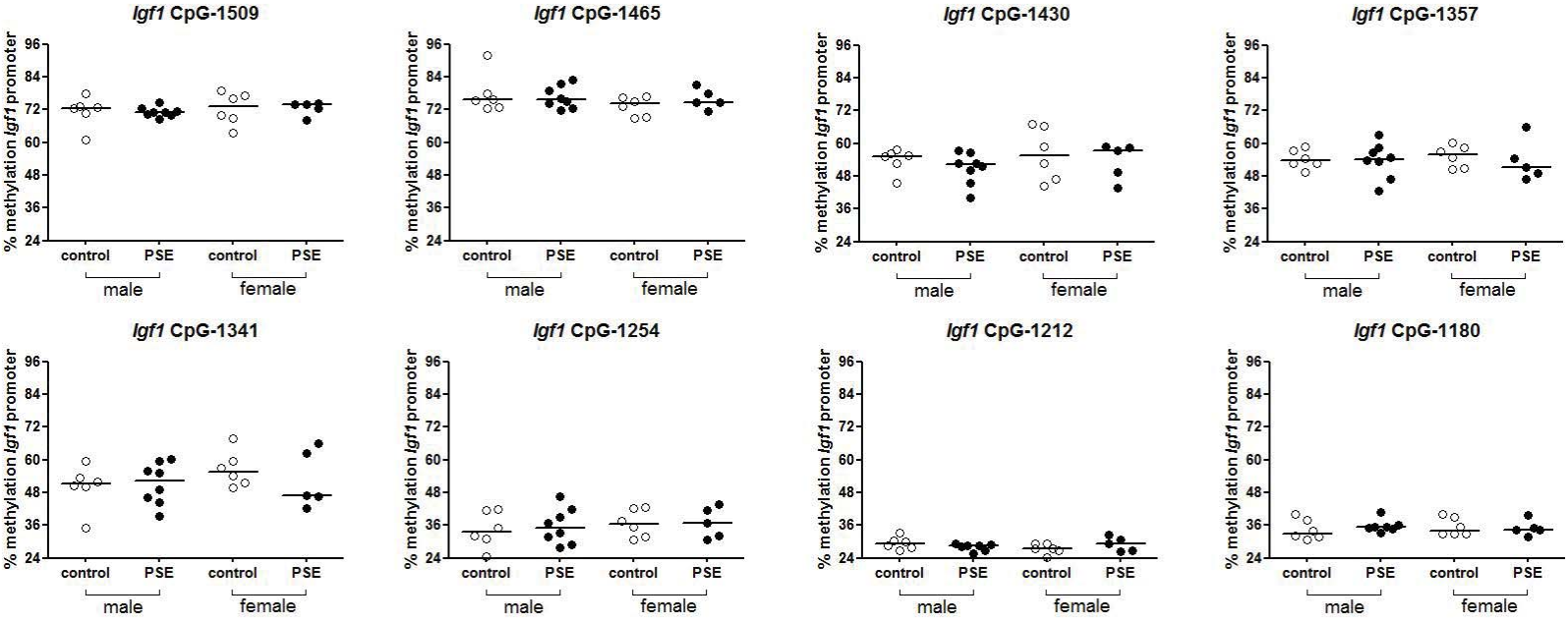
	CpG-166	r	0.01	0.06	0.08	0.04	-0.39	0.46	-0.40	-0.26	-0.90
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-164	r	0.27	0.07	0.22	-0.06	0.08	-0.34	-0.03	-0.23	0.22
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-17	r	**0.55**	**0.65**	0.59	0.53	-0.22	0.75	-0.04	0.50	-0.64
		p-value	**0.01**	**0.03**	0.07	0.09	ns	ns	ns	ns	ns
correlation of / with	**weight [g]**		all all	all male	all female	all control	all PSE	male control	male PSE	female control	female PSE
***Igf1r* promoter methylation [%]**	CpG-272	r	0.20	0.22	0.17	0.27	-0.57	-0.07	-0.77	0.38	-0.63
		p-value	ns	ns	ns	ns	0.07	ns	0.07	ns	ns
	CpG-255	r	0.05	0.17	-0.06	-0.09	-0.58	-0.22	-0.42	-0.19	-0.79
		p-value	ns	ns	ns	ns	0.06	ns	ns	ns	ns
	CpG-252	r	0.14	0.26	0.06	0.04	-0.45	0.11	-0.44	-0.14	-0.03
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-249	r	0.18	0.03	0.32	0.19	-0.28	-0.11	-0.54	0.21	0.43
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-246	r	0.22	0.30	0.06	0.25	-0.57	0.03	-0.65	0.11	-0.52
		p-value	ns	ns	ns	ns	0.07	ns	ns	ns	ns
	CpG-238	r	0.23	0.04	0.47	0.21	-0.19	-0.39	-0.47	0.46	0.38
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-233	r	**0.55**	0.47	0.47	0.33	**0.62**	-0.03	0.42	0.12	0.64
		p-value	**0.004**	ns	ns	ns	**0.04**	ns	ns	ns	ns
	CpG-230	r	0.22	0.04	0.26	0.24	-0.17	-0.19	-0.68	0.18	0.21
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-228	r	0.03	-0.18	0.14	0.43	-0.53	0.51	-0.66	0.49	-0.64
		p-value	ns	ns	ns	ns	0.09	ns	ns	ns	ns
	CpG-223	r	0.36	0.06	0.52	**0.54**	0.09	0.21	-0.59	0.66	0.48
		p-value	0.08	ns	0.07	**0.05**	ns	ns	ns	0.07	ns
	CpG-215	r	0.14	0.11	0.26	0.00	0.29	-0.29	0.29	0.20	0.02
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-209	r	-0.29	-0.01	-0.49	-0.26	-0.39	-0.45	-0.05	-0.40	-0.55
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-206	r	-0.30	-0.22	-0.11	-0.11	**-0.66**	-0.52	**-0.89**	0.20	-0.40
		p-value	ns	ns	ns	ns	**0.03**	ns	**0.02**	ns	ns
	CpG-201	r	-0.20	-0.37	0.04	-0.38	-0.33	-0.73	-0.73	-0.19	0.61
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-194	r	-0.28	0.06	-0.39	-0.02	-0.44	0.59	-0.27	-0.19	-0.25
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-185	r	-0.32	-0.14	-0.34	-0.38	-0.45	-0.40	-0.23	-0.35	-0.55
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-182	r	0.03	0.03	0.19	-0.01	0.14	0.21	0.35	0.47	0.08
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-171	r	-0.39	-0.42	-0.35	-0.39	-0.42	-0.79	-0.76	-0.44	-0.01
		p-value	0.06	ns	ns	ns	ns	0.06	0.08	ns	ns
	CpG-166	r	0.06	-0.02	0.13	-0.03	0.22	-0.53	0.32	0.27	0.03
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-164	r	-0.01	0.22	0.14	-0.04	-0.13	0.01	-0.12	0.19	0.20
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns
	CpG-17	r	0.07	-0.10	0.25	-0.19	0.05	-0.68	-0.14	0.03	0.02
		p-value	ns	ns	ns	ns	ns	ns	ns	ns	ns

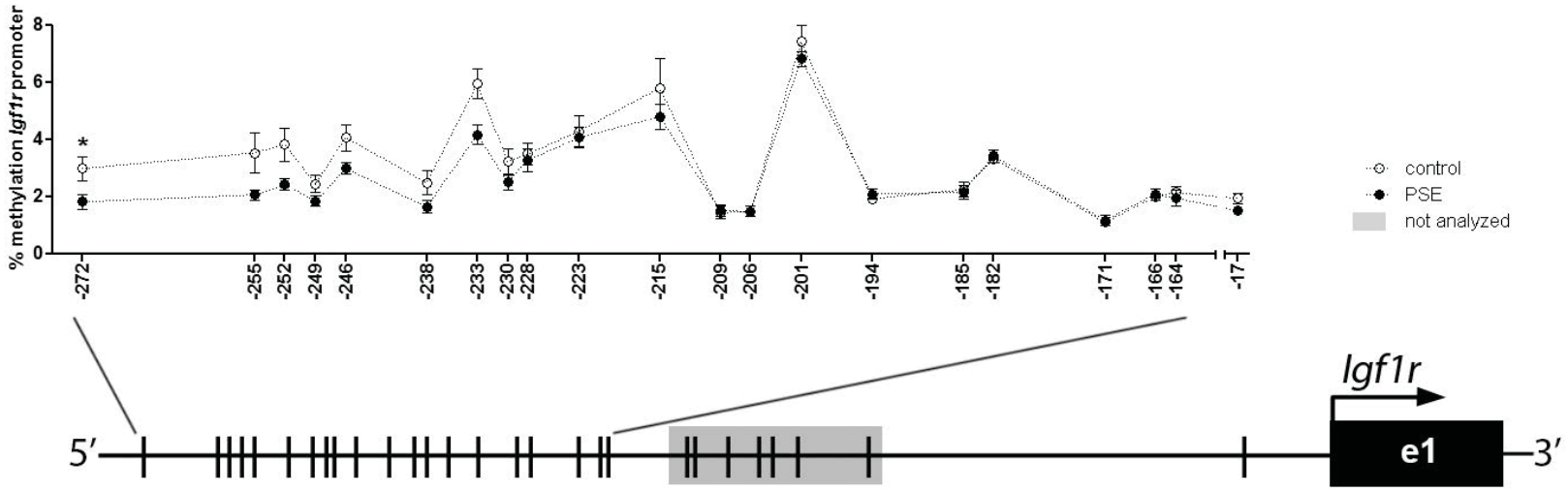

A
p = 0.04
p < 0.07
p = 0.05
weight [g]
20
18
16
14
12
10
control
PSE
control
PSE
male
female
B
IGF1 in whole lung (pg/ml)
10000
8000
6000
4000
2000
0
10
12
14
16
18
20
weight [g]
R2 = 0.29
p = 0.01
C
IGF1R in whole lung (pg/ml)
10000
8000
6000
4000
2000
0
10
12
14
16
18
20
weight [g]
R2 = 0.09
ns
○/ ● = male, control / PSE
□ / ■ = female, control / PSE

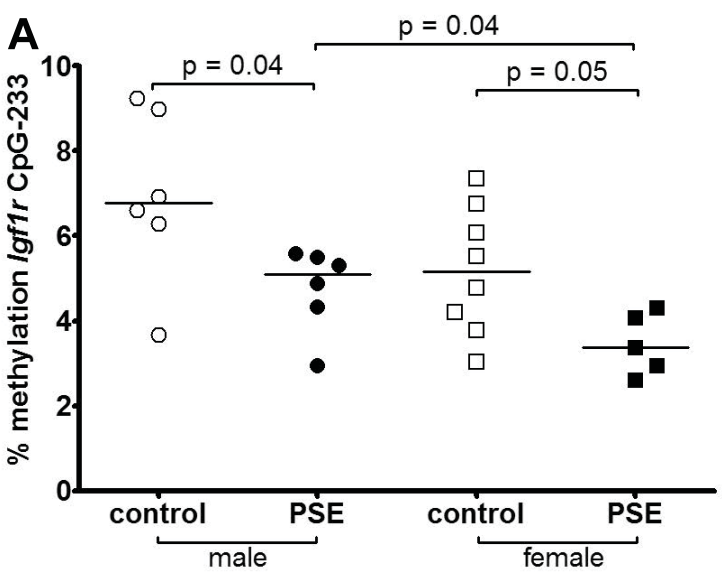

A
B
p = 0.05
p = 0.03
Igf1 (2-ΔCt)
Igf1r (2-ΔCt)
control
PSE
control
PSE
male
female
male
female

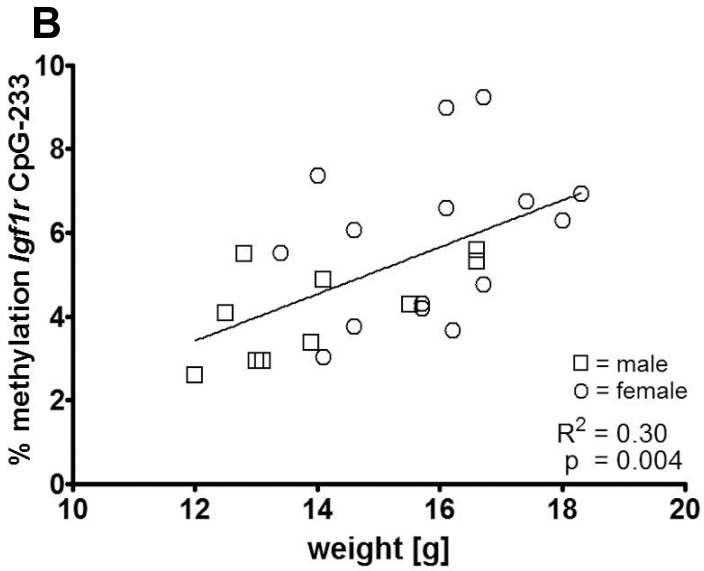

A
40
35
30
25
Igf1r (Ct)
25
30
35
40
Igf1 (Ct)
R2 = 0.91
p < 0.0001
B
5.0
4.5
2.5
2.0
1.5
1.0
0.5
0.0
Igf1 (2-ΔCt)
0
2000
4000
6000
8000
10000
IGF1 in lung tissue [pg/ml]
R2 < 0.01
ns
C
2.0
1.5
1.0
0.5
0.0
Igf1r (2-ΔCt)
0
2000
4000
6000
8000
10000
IGF1R in lung tissue [pg/ml]
R2 = 0.35
p = 0.02
○ / ● = male, control / PSE
□ / ■ = female, control / PSE

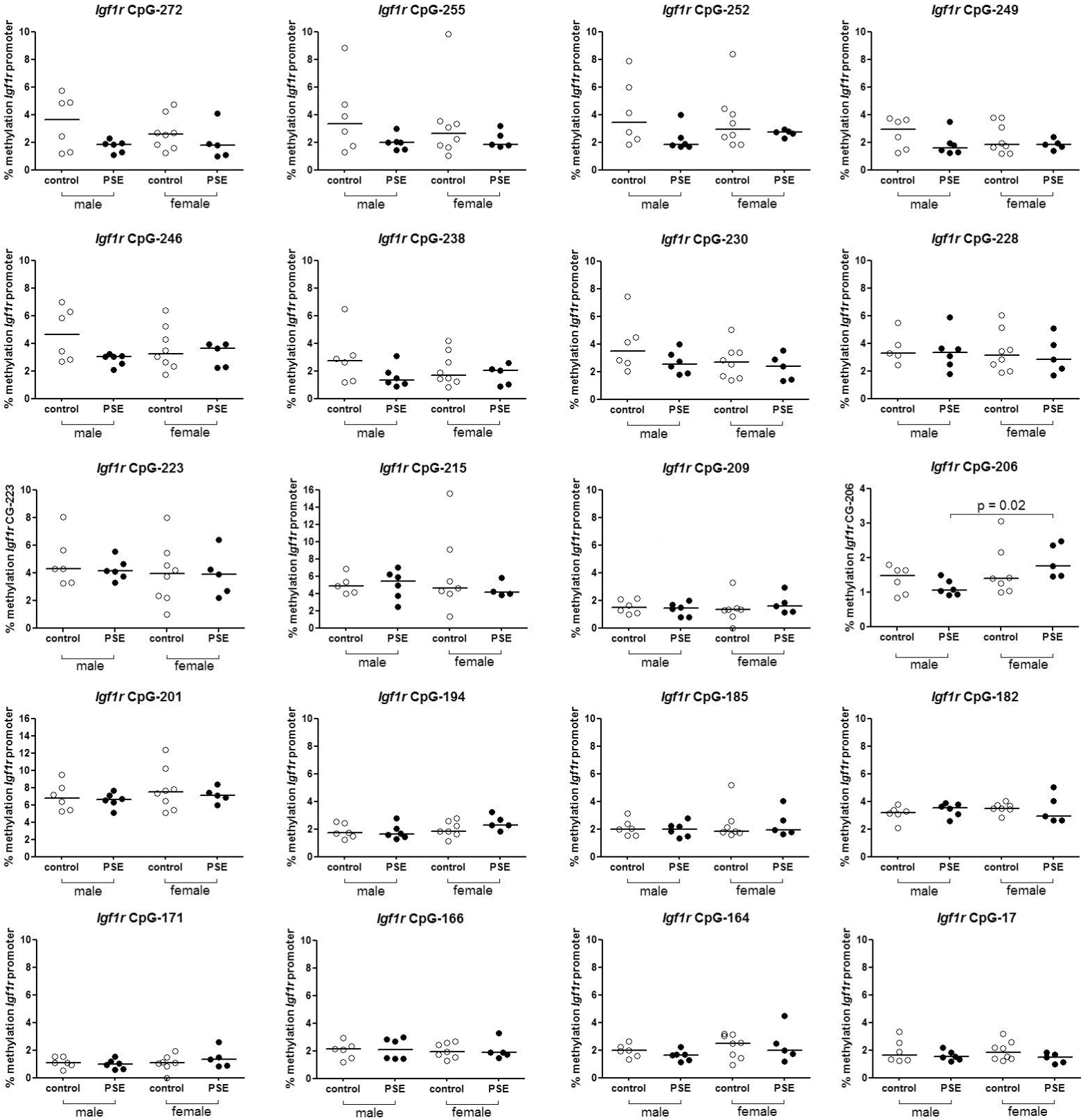

100
80
60
40
20
0
% methylation
-1509
-1465
-1430
-1357
-1341
-1254
-1212
-1180
control
PSE
Igf1
5'
5'UTR
e1
3'


Igf1 CpG-1509
Igf1 CpG-1465
Igf1 CpG-1430
Igf1 CpG-1357
Igf1 CpG-1341
Igf1 CpG-1254
Igf1 CpG-1212
Igf1 CpG-1180
% methylation *Igf1* promoter
96
84
72
60
48
36
24
control
PSE
male
female


% methylation lgf1r promoter
8
6
4
2
0
*
-272
-255
-252
-249
-246
-238
-233
-230
-228
-223
-215
-209
-206
-201
-194
-185
-182
-171
-166
-164
-17
control
PSE
not analyzed
lgf1r
5′
e1
3′


A
p = 0.04
p = 0.04
p = 0.05
% methylation Igf1r CpG-233
10
8
6
4
2
0
control
PSE
control
PSE
male
female


B
% methylation Igf1r CpG-233
10
8
6
4
2
0
10
12
14
16
18
20
weight [g]
□ = male
○ = female
R2 = 0.30
p = 0.004


lgf1r CpG-272
lgf1r CpG-255
lgf1r CpG-252
lgf1r CpG-249
lgf1r CpG-246
lgf1r CpG-238
lgf1r CpG-230
lgf1r CpG-228
lgf1r CpG-223
lgf1r CpG-215
lgf1r CpG-209
lgf1r CpG-206
p = 0.02
lgf1r CpG-201
lgf1r CpG-194
lgf1r CpG-185
lgf1r CpG-182
lgf1r CpG-171
lgf1r CpG-166
lgf1r CpG-164
lgf1r CpG-17
% methylation lgf1r promoter
% methylation lgf1r CG-223
% methylation lgf1r CG-206
control
PSE
male
female